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1 **Metabolomics reveals the physiological response of *Pseudomonas***
2 ***putida* KT2440 (UWC1) after pharmaceutical exposure.**

3

4 Running title: Metabolomics reveals abiotic perturbations to *P. putida*

5

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19

20

21 **Abstract**

22 Human pharmaceuticals have been detected in wastewater treatment plants, rivers, and
23 estuaries throughout Europe and the United States. It is widely acknowledged that there
24 is insufficient information available to determine whether prolonged exposure to low
25 levels of these substances is having an impact on the microbial ecology in such
26 environments. In this study we attempt to measure the effects of exposing cultures of
27 *Pseudomonas putida* KT2440 (UWC1) to six pharmaceuticals by looking at differences
28 in metabolite levels. Initially, we used Fourier transform infrared (FT-IR) spectroscopy
29 coupled with multivariate analysis to discriminate between cell cultures exposed to
30 different pharmaceuticals. This suggested that on exposure to propranolol there were
31 significant changes in the lipid complement of *P. putida*. Metabolic profiling with gas
32 chromatography-mass spectrometry (GC-MS), coupled with univariate statistical
33 analyses, was used to identify endogenous metabolites contributing to discrimination
34 between cells exposed to the six drugs. This approach suggested that the energy
35 reserves of exposed cells were being expended and was particularly evident on exposure
36 to propranolol. Adenosine triphosphate (ATP) concentrations were raised in *P. putida*
37 exposed to propranolol. Increased energy requirements may be due to energy dependent
38 efflux pumps being used to remove propranolol from the cell.

39

40 **Introduction**

41 Studies spanning the last 20 years, have shown that human pharmaceuticals are present
42 in waste water treatment plants (WWTPs), rivers and estuaries at the ng L⁻¹ or low µg L⁻¹
43 level throughout Europe and in the United States.¹⁻⁹ In monitoring studies in the UK 13
44 pharmaceuticals selected from priority lists of the UK Environment Agency and the
45 Oslo and Paris Commission (OSPAR) were detected at concentrations ranging from 11
46 – 69,570 ng L⁻¹ in raw WWTP effluent¹.

47 Wastewater from large industrial sites in India and China producing generic drugs in
48 bulk for the global market has been shown to be a source of far higher environmental
49 concentrations of pharmaceuticals¹⁰. Fick and colleagues showed contamination of
50 surface, ground and drinking water in the Hyderabad drug-producing area, where 9
51 drugs were detected in the mg L⁻¹ range in two lakes and at high ng L⁻¹ or low µg L⁻¹
52 levels in wells located in surrounding villages.¹¹ The effluent from the WWTP serving
53 approximately 90 bulk drug manufacturers shown to contain high levels of drugs with a
54 range of vertebrate drug targets was toxic to aquatic vertebrates even at high dilutions,
55 with 40% reduced growth in tadpoles in diluted (0.2%) effluent, and a median lethal
56 concentration for zebrafish between 2.7-8.1%.¹²

57 Pharmaceuticals merit concern as environmental pollutants because they are designed
58 with high potency and high specificity for interaction with biological systems: they are
59 of possible harm to the environment because they are designed to target specific
60 receptors/enzymes, which may have homologs in other species. Although studies such
61 as that of Gunnarsson *et al.*¹³ conclude that bacteria have both low numbers of
62 homologs and low sequence similarity to those in man, many microbial organisms have
63 an important environmental role which includes element cycling and the degradation of
64 xenobiotic pollutants, and stress responses due to chronic exposure might impact on

65 fitness for survival. While Sumpter in his recent review¹⁴ concludes that the vast
66 majority of pharmaceuticals will have no appreciable real-world environmental
67 consequence it has been shown that pharmaceuticals have a range of acute and chronic
68 toxicities on test organisms where environmental exposure is persistent at low
69 concentrations.^{15, 16} It has been suggested that both *concentration addition*, where
70 multiple compounds of the same class are present, each at low concentration, and
71 *response addition* where multiple compounds of different classes are present, may cause
72 stress responses in organisms and impact on fitness for survival.¹⁷⁻²⁰ Environmental risk
73 assessments (ERA) are now required when applying for marketing authorization for a
74 medicinal product for human use in the European Union (EU) (Council Directive
75 2001/83/EC as amended by Council Directive 2004/27/EC). In a tiered approach to
76 testing, evaluation beyond acute toxicity tests is required where the predicted
77 environmental concentration (PEC) of a pharmaceutically active compound is more than
78 0.01 µg L⁻¹. There is clearly a need to ascertain chronic effects of pharmaceuticals in the
79 environment, and to mitigate pollution due to bulk drug manufacture.

80

81 Here, we studied the effects of pharmaceutical exposure on *Pseudomonas putida*, a
82 metabolically versatile soil bacterium, able to undertake important metabolic activities
83 in the environment, including element cycling and the degradation of xenobiotic
84 pollutants.²¹ *P. putida* strain KT2440 has been certified as a biosafety host for the
85 cloning of foreign genes, and has potential for biotechnological applications such as
86 bioremediation and biocatalysis.²² *P. putida* KT2440 UWC1 is a plasmid-free,
87 restriction-negative, spontaneous rifampin-resistant derivative of *P. putida* KT2440.²³
88 Recent sequencing of the 6.1Mb genome of *P. putida* KT2440²⁴ has revealed diverse

89 metabolic and transport systems, with a comparatively high number of efflux pumps
90 associated with protection against toxic substrates and metabolites.

91 Metabolomics is a well-established field for the global analysis of endogenous
92 metabolites within cells, tissue, biofluids, organs or whole organisms, and utilizes
93 analytical techniques combined with statistical analysis. Where traditional studies may
94 focus on one or a few biochemical pathways, discovery metabolomics attempts to
95 observe global metabolite alterations. As such, discovery metabolomics is considered to
96 be hypothesis generating, rather than hypothesis testing, with the possibility of finding
97 novel results which should be tested further targeted experiments. Metabolomics offers
98 several distinct advantages over other omic studies. A ten-fold difference is generally
99 observed in the number of metabolites compared to the number of genes (fewer
100 metabolites than genes), making the metabolome more amenable to both chemical
101 analysis and data interpretation.^{25, 26} While alterations in the transcriptome or proteome
102 may not always lead to changes in the metabolic phenotype²⁷, the metabolome
103 represents the final products of gene expression and is closest to the function or
104 phenotype of the cell. Furthermore, metabolic control analysis (MCA) has
105 demonstrated that changes in concentration of metabolites can be observed even when
106 alterations in the concentrations of transcripts and proteins are small.²⁸ Metabolomics is
107 a high-throughput strategy with low costs per analysis compared to transcriptomic and
108 proteomic technologies, and, unlike other omics techniques, does not rely on species-
109 specific information. The reader is directed to the literature for information on the
110 analytical technologies and methodologies²⁹⁻³⁶, and on the statistical approaches used in
111 metabolomics.³⁷ Environmental metabolomics has recently been defined as the
112 application of metabolomics techniques to characterise the metabolism of free living
113 organisms obtained from the natural environment and of organisms reared under

laboratory conditions, where those conditions specifically serve to mimic scenarios encountered in the natural environment.³⁸ There is considerable potential for omic profiling methods to progress significant advances in regulatory ecotoxicology, with applications including biomarker development and risk assessment for toxicant exposure, and evaluation of metabolic responses to environmental stressors.^{39, 40} Reviews of recent applications in environmental metabolomics can be found in^{41, 42}.

In whole organism fingerprinting FT-IR spectroscopy measures bond vibrations of functional chemical groups in cell constituents such as DNA and RNA, proteins, lipids and carbohydrates. FT-IR may be used as a screening tool providing rapid discrimination between samples, through measurement of overall phenotypic changes in a sample without specific identification of the individual metabolites responsible. In contrast, metabolic profiling by GC-MS provides semi-quantification and, where possible, the definitive identification of metabolites through retention time and mass spectrum matching. Subsequent data analysis can then reveal discriminatory metabolites.³³

In this study we monitored the effect on the metabolism of *P. putida* KT2440 UWC1 of exposure to six pharmaceutical compounds; four analgesics (3 non-steroidal anti-inflammatory drugs (NSAID) and acetaminophen - a possible COX -isoform inhibitor but with an ill-defined mechanism of action), and two β -adrenergic receptor agonists (Table SI3 1, Supplementary Information). Five of the compounds acetaminophen, diclofenac, ibuprofen, mefenamic acid and propranolol have been detected in wastewater treatment plant effluent in the UK.¹ Acetaminophen was detected in raw effluent only at a mean concentration of 27,341 ng L⁻¹, diclofenac throughout the treatment plant at concentrations ranging from 342-978 ng L⁻¹, ibuprofen 3063-23,161

139 ng L⁻¹, mefenamic acid 234-959 ng L⁻¹ and propranolol 83-291 ng L⁻¹. Roberts and
140 Thomas suggest that it would also be beneficial to determine levels of parent
141 compounds present in sewage sludge, in order to determine levels of adsorption.¹

142 In this study we exposed *P. putida* KT2440 to the pharmaceuticals at a single
143 concentration of 50 µg mL⁻¹; although higher than measured environmental
144 concentrations in the UK, this is a concentration well below the minimum inhibitory
145 concentrations established for the pharmaceuticals, and at which we had seen a
146 measurable effect in earlier experiments using FT-IR spectroscopy. We performed a
147 principal components-canonical variates analysis on the FT-IR spectra of whole cells,
148 and ANOVA and correlation analysis on the GC-MS profiles of the methanol cell
149 extracts of *P. putida* exposed either to one of the pharmaceuticals or to water as a
150 control. Metabolic fingerprinting by FT-IR spectroscopy suggested that on exposure to
151 propranolol there were significant changes in the lipid complement of *P. putida*.
152 Metabolic profiling from GC-MS measurements suggested that the energy reserves of
153 exposed cells were being expended and this was particularly evident on exposure to
154 propranolol. Therefore we measured adenosine triphosphate (ATP) concentrations in *P.*
155 *putida* exposed to propranolol using a bioluminescence assay.

156

157 **Materials and Methods**

158 **Materials and Methods for the experiment and for the statistical analysis** are
159 described in detail in the Supplementary Information. In preliminary experiments the
160 effect of each pharmaceutical on growth of *P. putida* was determined, the minimum
161 inhibitory concentration (MIC) of the pharmaceuticals for *P. putida* KT2440 UWC1
162 was estimated, and the recovery of the pharmaceuticals was monitored by HPLC in

163 order to assess if there was any metabolism of the pharmaceuticals by *P. putida* over 24
164 h. For the metabolomics analysis, briefly, *Pseudomonas putida* KT2440 UWC1 was
165 cultured, in replicate, in liquid medium supplemented with one of 6 drugs at a
166 concentration of 50 $\mu\text{g mL}^{-1}$, or water as a control. At the end of the exponential growth
167 period cells were harvested and the sample split to provide cells for FT-IR spectroscopy
168 and for GC-MS. Metabolite fingerprinting of whole cells by FT-IR spectroscopy was
169 carried out according to a modified method of Goodacre *et al.*⁴³ Metabolite profiling of
170 methanol cell extracts by GC-MS was carried out according to a modified method of
171 Winder *et al.*³⁰ using GC-MS conditions optimized for yeast.⁴⁴ ATP in methanol:water
172 extracts of *P. putida* exposed to propranolol was measured using a bioluminescence
173 assay kit available from Roche Molecular Biochemicals (Roche Diagnostics, Burgess
174 Hill UK). Full details are given in the supplementary material. A combined principal
175 components-canonical variates analysis (PC-CVA) was carried out for both the FT-IR
176 spectra and GC-MS data using programs written in MATLAB⁴⁵ as detailed elsewhere.⁴⁶
177 ANOVA was carried out on GC-MS data using programs written in MATLAB
178 (<http://www.mathworks.com/>) and described elsewhere.³⁷ Correlation analysis for
179 metabolomics data is described by Steuer.^{47, 48} Correlation analysis for significantly
180 altered metabolites was carried out using Graphviz open source graph visualization
181 software⁴⁹ following an approach proposed by Kamada and Kawai.⁵⁰ Full details are
182 given in the supplementary material.

183 **Results and Discussion**

184 Results for the determination of minimum inhibitory concentrations, the effect of each
185 pharmaceutical on growth and monitoring recovery of the pharmaceuticals by HPLC are
186 given in the Supplementary Information.

187 *Multivariate Analysis of FT-IR Data:* A total of 26 PCs were extracted for a cross-
 188 validated PC-CVA model for the FT-IR spectra. Figure 1a shows the PC-CV score 1
 189 plotted against PC-CV score 2 for the FT-IR spectra of *P. putida* exposed to the six
 190 pharmaceuticals. In this analysis, the FT-IR spectra from 4 replicate cultures of *P.*
 191 *putida* were used as a training set, and the spectra from the fifth replicate were used as
 192 an independent test set with no *a priori* knowledge of the class structure. The test data
 193 should lie within the bounds of the training data, defined here as the 95% confidence
 194 limit from the group centres here constructed around each group mean by the χ^2
 195 distribution on two degrees of freedom, as observed for *P. putida* exposed to ibuprofen
 196 and mefenamic acid. The cells exposed to propranolol and ibuprofen are separated, both
 197 from the control and cells exposed to the remaining pharmaceuticals, along PC-CV1,
 198 and those exposed to mefenamic acid along PC-CV2. No effect on *P. putida* exposed to
 199 acetaminophen, atenolol or diclofenac was observed on inspection of the lower
 200 canonical variates (data not shown). Examination of the loadings for PC-CV1 from the
 201 PC-CV analysis (Figure 1b) shows, firstly, significantly high loadings occurring at
 202 several wavenumbers for propranolol at 1570, 1483, 1271, 1242, and 1102 cm^{-1} . High
 203 loadings in the region corresponding to aliphatic C-H, and hence bacterial fatty acids, at
 204 2919 and 2850 cm^{-1} prompted us to investigate lipid alterations in *P. putida* exposed to
 205 propranolol. There are also significantly high loadings in the regions corresponding to
 206 the amide I bands in protein structures at 1655 cm^{-1} (α -helical structures), 1709, 1659
 207 and 1630 cm^{-1} (β -sheet structures).⁵¹
 208 These observations, together with the reduction in free amino acids observed in the
 209 GC-MS analysis (*vide infra*) are consistent with the theory that cell integrity is
 210 maintained through *cis*-to-*trans* isomerization of membrane lipids which results in a

211 more rigid cell membrane structure, and synthesis of drug efflux pumps in order to
212 remove toxic substances from the cell.⁵²

213 The PC-CVA was repeated using absorbances at wavenumbers selected from those with
214 significantly high loadings shown in Figure 1b. Rebuilding the model with absorbances
215 only at wavenumbers significant for bacterial fatty acids showed discrimination between
216 *P. putida* exposed to propranolol and the control, and no discrimination of any other
217 exposure from the control (Figure 2). Thus, observed lipid alterations were specific for
218 exposure to propranolol. PC-CVA models built using absorbances at selected
219 wavenumbers significant for the fingerprint region showed little difference from the
220 model using the entire dataset, affording no new information, while there were too few
221 wavenumbers significant for protein to use successfully in a PC-CVA model.

222

223 (Figure 1)

224 (Figure 2)

225

226 *Univariate Analysis of GC-MS Data:* ANOVA was performed for *P. putida* exposed to
227 each pharmaceutical versus the control using the family-wise error rate (FWER) to
228 determine a suitable threshold for the p-value.⁵³ Thresholds equivalent to $\alpha = 0.05$ were
229 determined for cells exposed to propranolol (0.0177), diclofenac (0.006),
230 acetaminophen (0.005), atenolol (0.003) and mefenamic acid (1.76×10^{-4}). A threshold
231 was determined equivalent to $\alpha = 0.1$ for cells exposed to ibuprofen (0.013), in order to
232 be able to compare alterations in metabolites, since discrimination of these samples was
233 earlier observed from the FT-IR data. A total of 76 metabolites were significantly
234 altered overall with p-values below the FWER thresholds, and 67 of these had an area
235 under the ROC curve⁵⁴ > 0.85 . Of these, 43 were altered on exposure to propranolol, 17

236 on exposure to diclofenac, 16 on exposure to ibuprofen, 14 on exposure to
237 acetaminophen, 8 on exposure to atenolol, and 3 on exposure to mefenamic acid.
238 Metabolites are listed in the supporting information (Table SI2 1.), together with the p-
239 value and fold difference in median GC-MS peak response. In order to view alterations
240 which are common to exposure to the different pharmaceuticals, the 67 metabolites are
241 ordered firstly by significance (p-value) for exposure to propranolol, followed by
242 significance for exposure to diclofenac, ibuprofen, acetaminophen etc.

243 Metabolite identification is currently recognised as a major limitation in GC-MS
244 metabolomics studies, and a number of studies report metabolites of biological interest
245 as unidentified.⁵⁵ The accurate identification of metabolites requires the construction of
246 mass spectral / retention index libraries. Commercially available GC-MS libraries such
247 as NIST/EPA/NIH and Wiley have not been developed with the objective of including
248 endogenous (or exogenous) metabolites, and are not widely applicable in metabolomics
249 studies. A number of research groups have, therefore, developed their own metabolite
250 libraries employing both the mass spectrum and retention index to define a metabolite,
251 for example the Golm (<http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/gmd.html>),⁵⁶ and
252 Feihn (<http://fiehnlab.ucdavis.edu/Metabolite-Library-2007/>);⁵⁷ databases. For definitive
253 metabolite identification, comparison of fragmentation mass spectra with authentic
254 chemical standards is required. Standards are often unavailable for endogenous
255 metabolites and therefore only possible or probable identifications can be assigned in a
256 large number of instances. In this study those metabolites where the identification is
257 definitive are labeled with the metabolite name and an asterisk in the supplementary
258 information, those where the identification is tentative labeled with the metabolite
259 name, while unidentified metabolites are identified with a number. The recorded fold

260 difference is the median fold difference in GC-MS peak response (cells exposed to a
261 pharmaceutical / control cells exposed to water).

262 Altered metabolites in *P. putida* exposed to propranolol are visualised in Figure 3,
263 where the area under the ROC curve is plotted *versus* the p-value from the ANOVA
264 analysis. Metabolites with an area under the ROC curve > 0.7 and a p-value $< 2 \times 10^{-2}$
265 are labeled, and metabolites with an area under the ROC curve > 0.85 , with $p < 0.01$
266 were considered significant and selected for correlation analysis.

267 (Figure 3)

268 (Figure 4)

269 Spring-embedded correlation plots for the significant metabolites identified from the
270 ANOVA analysis show correlations in the cells exposed to water (Figure 4a) and
271 correlations in the cells exposed to propranolol (Figure 4b). Correlation analyses for *P.*
272 *putida* exposed to the other pharmaceuticals were not as informative as that for
273 propranolol and are not shown; for example in *P. putida* exposed to ibuprofen
274 correlations were observed only between trehalose and metabolites 35 and 47, and
275 further to 2-aminoethyldihydrogenphosphate and tropic acid, and between cellobiose,
276 putrescine, glycerol-3-phosphate a sugar alcohol and metabolite 95.

277

278 In *P. putida* exposed to propranolol 9 metabolites identified with the low p-values in the
279 ANOVA analysis ($< 5.6 \times 10^{-4}$) were raised in concentration from, or lowered in
280 concentration to, near the analytical limit of detection in exposed cells (Figure SI3 2 in
281 the supplementary information). All except metabolites 130 and 131 had an area under
282 the ROC curve of 1, implying that these metabolites are entirely diagnostic of cells
283 exposed to propranolol. Propranolol itself was present in the extracts of exposed cells
284 and absent in the control cells. The concentration of metabolites 129 and 130 (for which

285 there was no definitive identification) fell below the limit of detection in exposed cells.
286 Metabolite 129 was also altered significantly in cells exposed to diclofenac and
287 mefenamic acid. Metabolites 131, 134, 135, 139, 142, and 145 were raised in exposed
288 cells from near or below the limit of detection in the control, and were not present in
289 cells exposed to any other pharmaceutical in the study. Identification was not possible
290 from the mass spectra which contained only low m/z ions. Further work using ¹³C-
291 labeled propranolol would determine whether propranolol is metabolized by *P. putida*,
292 and whether metabolites 139, 134, 135, 142 and 145 are endogenous metabolites or
293 products of propranolol catabolism.

294

295 In *P. putida* exposed only to water, metabolite 130 is correlated with the amino acid
296 valine, and correlation extends through other amino acids to trehalose and cellobiose.
297 This correlation is disrupted within the sample set of *P. putida* exposed to propranolol
298 as the concentration of metabolite 130 falls to near to the limit of detection. In cells
299 exposed to propranolol the concentrations of trehalose and cellobiose are lowered by 0.5
300 and 0.8, respectively, when compared to *P. putida* exposed only to water. In *P. putida*
301 exposed to propranolol, propranolol itself is correlated to both trehalose and cellobiose
302 and another energy related metabolite, a sugar phosphate, and correlation is extended
303 from propranolol through cellobiose and metabolite 139 to succinic acid, a metabolite of
304 the TCA cycle. Concentrations of sugar phosphates and succinic acid were raised, 1.3 -
305 2.1 fold and 3 fold, respectively, in *P. putida* exposed to propranolol, suggesting
306 increased glucose flux through glycolysis, as does utilization of trehalose and cellobiose
307 (Figure 5).

308 (Figure 5)

309 Glycolysis is primarily via the pentose phosphate and the Entner-Doudoroff pathways in
310 *Pseudomonas*.⁵⁸ Trehalose degradation to glucose is inferred in *P. putida* via the
311 pathway trehalose degradation I, from its expected taxonomic range, in the MetaCyc
312 database.⁵⁹ We observed a 1.3 fold increase in xylitol in exposed cells indicative of
313 pentose interconversions.

314

315 Both trehalose and cellobiose were identified as significantly altered metabolites on
316 exposure to more than one pharmaceutical: in *P. putida* exposed to propranolol,
317 ibuprofen, acetaminophen and atenolol the concentration of trehalose was lowered
318 significantly by 0.5, 0.4, 0.4, and 0.5; in *P. putida* exposed to propranolol and ibuprofen
319 the concentration of cellobiose was also lowered significantly by 0.8 and 0.3,
320 respectively. Trehalose has been observed to have a role as an osmoprotectant; for
321 example trehalose concentration in *P. aeruginosa* was recently shown to be dependent
322 on NaCl concentration⁶⁰, and in response to solvent exposure in *Pseudomonas* sp.
323 BCNU171.⁶¹ In this study the concentration of both trehalose and cellobiose were
324 reduced and we inferred from these changes that the disaccharides trehalose and
325 cellobiose, which have a role as an energy reserve (both converted to D-glucose), are
326 utilized as some energy consuming process(es) is (are) upregulated on exposure to
327 propranolol. We confirmed that ATP concentrations were raised in *P. putida* exposed to
328 propranolol (*vide infra* and Figure 6). Propranolol, which has a planar naphthalene
329 structure and is known to interact with lipid membranes⁶², is present in the extracts of
330 the exposed cells. It may be that an energy dependent efflux pump is one mechanism
331 used to remove propranolol from the cell. *P. putida* KT2440 genome sequencing²⁴
332 revealed a large number of different efflux systems that may be involved in the active
333 export of solvents, and the TolC outer membrane channel protein has been shown to be

334 upregulated in response to phenol.⁶³ Concurrent with solvent extrusion, a process with
335 high energy demand partly required for the operation of efflux pumps, sugar uptake,
336 glucose catabolic enzymes, and TCA cycle enzymes are induced.⁶⁴

337

338 Amino acids were reduced in concentration on exposure to propranolol by 0.29 – 0.73
339 fold (Table SI3 2 in the supplementary information.). By contrast, few significant
340 alterations in amino acids were observed on exposure to other pharmaceuticals.
341 Alterations were observed on exposure to ibuprofen (phenylalanine: 1.2 fold increase),
342 diclofenac (β -alanine: 0.73 fold decrease) and acetaminophen (phenylalanine: 0.2 fold
343 decrease, and glutamic acid: 0.72 fold decrease). Together with alterations in the protein
344 complement of exposed cells observed in the FT-IR analysis this is consistent with *de*
345 *novo* protein synthesis (possibly including an efflux system) in response to exposure.
346 Using DNA array technology to investigate the response of *P. putida* KT2440 to
347 toluene, o-xylene and 3-methylbenzoate Dominguez-Cuevas and co-workers observed
348 major changes in genes related to amino acid biosynthesis and critical functions for
349 protein production.⁶⁵ Strong induction of methionine biosynthesis was observed. In
350 addition, leucine, isoleucine, tryptophan, serine and arginine biosynthesis was induced
351 while catabolism of tryptophan and arginine was turned down, reflecting the need for
352 amino acids in the new proteome found in cells exposed to toluene.

353

354 In *P. putida* exposed to propranolol we observed a significant 0.66 fold decrease in
355 myoinositol which has a role in glycosylphosphatidylinositol (GPI)-anchor biosynthesis,
356 and the anchoring of cell-surface proteins to the cell membrane.

357

358 In *P. putida* exposed to propranolol we observed that the concentrations of 2-
359 monopalmitin and monostearin were lowered to near the analytical limit of detection
360 and by 0.8, respectively. The level of octadecanoic acid was raised 8 fold, although the
361 p-value from the ANOVA was marginally greater than the threshold p-value at $2.14 \times$
362 10^{-2} . We observed a significant 2 fold increase in levels of glycerol-3-phosphate which
363 has a major role in glycerolipid and glycerophospholipid metabolism, where it is the
364 precursor to the phosphatidyl moiety and the two phosphatidyl residues linked by a
365 glycerol moiety in cardiolipins. We observed a significant 0.49 fold decrease in level of
366 heptadecanoic acid decreased, and a slight increase (1.15 fold) in the level of
367 pentadecanoic acid. Other fatty acids detected were hexadecanoic, hexadecenoic, *cis*-9-
368 octadecenoic acid, and octadecenoic acid methyl ester and were not altered significantly
369 on exposure to propranolol.

370

371 In cells exposed to diclofenac, atenolol and mefenamic acid, the concentration of 9-
372 octadecenoic acid methyl ester fell to the analytical limit of detection, and hexadecenoic
373 acid lowered by 0.9 in cells exposed to ibuprofen and may be indicative of
374 cyclopropane fatty acid formation. The unsaturated fatty acids *cis*-9-octadecenoic, *cis*-
375 11-octadecenoic and *cis*-9-hexadecenoic acids are the precursors of the cyclopropane
376 fatty acids found in *E. coli* and *P. putida*, *cis*-9,10- and *cis*-11,12-
377 methyleneoctadecanoic (C₁₉) and *cis*-9,10-methylenehexadecanoic (C₁₇) acids ⁶⁶.
378 However, methylation is to the esterified fatty acid in phospholipids (the C1 donor is S-
379 adenoyslmethionine), and, since the extraction protocol for GC-MS did not allow
380 detection of fatty acids from esterified lipids, we were unable to detect any
381 corresponding alterations in C₁₉ or C₁₇ cyclopropane fatty acids. The concentration of
382 pentadecanoic acid increased 1.1 fold in cells exposed to ibuprofen and acetaminophen.

383

384 Ramos and colleagues summarized several alterations in fatty acid composition in the
385 bacterial response to solvent exposure⁵², which include *cis* to *trans* isomerization of
386 esterified fatty acids, a shift in the ratio of saturated : unsaturated fatty acids and
387 formation of C₁₇ cyclopropane fatty acids. Quantitative proteomics has revealed the
388 upregulation of proteins involved in cell wall biosynthesis and plasma membrane fatty
389 acids, and the outer membrane efflux protein TolC in the phenol-induced stress-
390 response in KT2440.⁶³ The highest level of phenol-stimulation was observed for AccC-
391 1. This is the enzyme encoding the first step of the fatty acid biosynthetic pathway and
392 leads to an increase in the rate of fatty acid biosynthesis under phenol stress as a
393 recovery mechanism for oxidatively damaged membrane phospholipids.

394 Propranolol is known to interact with lipid membranes and was observed in the
395 methanol extracts of exposed cells. Tolerance to toluene in *P. putida* DOT-T1E has
396 been suggested to be based on its exclusion by constitutive and inducible efflux pumps
397 and rigidification of the cell membranes via phospholipid alterations.⁶⁴ A number of
398 studies have looked at adaptive changes in membrane lipids in response to solvent
399 exposure. Studies by Junker and Ramos showed that a major adaptive change observed
400 in the solvent resistant strain *Pseudomonas putida* DOT T1E in response to solvent is
401 *cis* to *trans* isomerization in membrane lipids, predominantly in
402 phosphatidylethanolamines, which counteracts the increase in membrane fluidity caused
403 by toluene.⁶⁷ The *cis:trans* ratio decreased from 7.5 to 1 when cells were grown in 1%
404 toluene and changes were observed within 1 min of solvent exposure. The isomerase cti
405 is located in periplasm where access to esterified phospholipids is possible and *cis* to
406 *trans* isomerization is the main adaptive change in the short term, allowing cells to
407 adapt immediately to environmental conditions in which a denser membrane packing is

408 a selective advantage. Cells gain time for *de novo* biosynthesis of membrane
409 components as late as 15 min after solvent exposure. These changes include a shift in
410 the ratio of saturated : unsaturated fatty acids and formation of C₁₇ cyclopropane fatty
411 acids, synthesis of solvent extrusion pumps, modifications in lipid polysaccharides and
412 alterations in membrane protein content.

413

414 *ANOVA analysis of ATP concentrations prior to and post exposure to propranolol:* We
415 observed a significant rise in ATP concentration from 3.22 to 4.10 moles mg⁻¹ dry
416 weight cells in *P. putida* after 1 h exposure to propranolol (Figure 6).

417

418 The critical p-value (α) here was assumed to be 0.01. p-values were calculated in an
419 ANOVA analysis for the null hypothesis that the medians of the 2 groups are equal: the
420 p-value for controls prior to exposure vs. controls 1 h after exposure = 1.2×10^{-1} and the
421 p-value for exposed cells prior to exposure vs. 1 h after exposure = 1.2×10^{-5} , showing
422 a significant difference at the $\alpha = 0.01$ level in ATP concentration and energy demand in
423 cells exposed to propranolol.

424

425 (Figure 6)

426 **Conclusions**

427 In conclusion, we have presented a novel metabolomics approach to investigate the
428 effect of human pharmaceuticals on the environmentally relevant microorganism *P.*
429 *putida* KT2440 (UWC1). Metabolic profiling using GC-MS coupled with univariate
430 analysis and spring embedded correlation analysis was used to identify metabolites
431 contributing to discrimination between cells exposed to the six drugs, and statistically

432 significant differences were observed for propranolol, diclofenac, ibuprofen and
433 acetaminophen compared to untreated control cells.

434

435 The concentrations of several metabolites were altered significantly on exposure to a
436 number of the pharmaceuticals and may be considered biomarkers of abiotic stress. The
437 endogenous, metabolites 129 and 130 were significantly reduced in concentration in *P.*
438 *putida* exposed to propranolol, and 129 was reduced in cells exposed to diclofenac and
439 mefenamic acid. Concentrations of trehalose and metabolite 47 were also significantly
440 reduced on exposure to propranolol, ibuprofen, acetaminophen and atenolol.

441

442 Six metabolites, 131, 134, 135, 139, 142, and 145, were raised in exposed cells from
443 near or below the limit of detection in the control, and were not present in cells exposed
444 to any other pharmaceutical in the study. Identification was not possible from the mass
445 spectra which contained only low m/z ions. Further work using ^{13}C -labelled propranolol
446 would determine whether propranolol is metabolized by KT2440, and whether these
447 metabolites are endogenous metabolites or products of propranolol catabolism.

448

449 We also note that the growth conditions we have used (*viz.* R2A medium) are
450 considerably more nutrient rich than what would normally be expected in an
451 environmental water sample or found within benthic-sediment ecology (although in the
452 benthos or fresh water sediment one would expect the APIs to be more concentrated).
453 Future work would be to investigate the use of the above markers of abiotic stress in a
454 suitable ecosystem. Such an approach would involve target metabolite analysis
455 encompassing significant sample clean up, specific metabolite extraction and targeted
456 MS-MS for definitive metabolite identification and quantification.

457

458 With respect to exposure to propranolol, FT-IR analysis revealed changes in fatty acids
459 and protein structure while GC-MS revealed alterations in energy reserves, amino acids
460 and some fatty acids. Measurement of ATP concentrations in *P. putida* exposed to
461 propranolol showed an increased level of ATP in exposed cells. These alterations are in
462 agreement with previous studies which have shown that lipids in the membrane are
463 altered to try to retain membrane integrity, and that energy dependent efflux pumps are
464 used to remove toxic compounds from the cell. Additional studies undertaken by us
465 include further investigation of the phospholipid and fatty acid alterations in *P. putida*
466 exposed to propranolol, and these will be reported elsewhere.

467

468 We believe that this approach shows for the first time the value of developing a
469 comprehensive metabolomics-based approach both for identifying discriminatory
470 metabolites and their relationships to each other that reproducibly alter under abiotic
471 stress. Moreover, this approach allows the investigation of mechanisms of response to
472 these stresses in environmentally relevant microbes and future work will investigate
473 these effects in complex microbial communities.

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478

479 **Conflict of Interests**

480 All authors have no conflict of interest to declare.

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484 Supplementary information is available online
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486

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Figure Legends

Figure 1.

a) Cross-validated PC-CVA models for the FT-IR spectra of *P. putida* exposed to the six pharmaceuticals.

The FT-IR spectra were normalized and variables scaled to unit variance. 26 PCs (99.99% explained variance) and 6 CVs were used in the analysis trained on 4 class replicates (filled triangles). Test data (a fifth, unknown, class replicate) are marked with an asterisk (open triangles). Key; acetaminophen red, atenolol gold, diclofenac green, ibuprofen cyan, mefenamic acid blue, propranolol purple, control grey. Circles represent the 95% confidence limit from the group centres here constructed around each group mean by the χ^2 distribution on two degrees of freedom. Cells exposed to propranolol and ibuprofen are separated along PC-CV1; those exposed to mefenamic acid are separated along PC-CV2.

b) Examination of the loadings for PC-CV1 from the PC-CV analysis.

Significant loadings with amplitude $>2SD$ from the mean are shown in blue overlaid with the FT-IR spectrum for propranolol (green) and the FT-IR spectrum for the cells exposed to propranolol at $50\mu\text{g mL}^{-1}$ (blue). Several high loadings for PC-CV1 occur at significant wavenumbers for propranolol, the region corresponding to aliphatic C-H, and hence bacterial fatty acids, at 2919 and 2850cm^{-1} , and the regions corresponding to the amide I bands in protein structures at 1655cm^{-1} (α -helical structures), 1709 , 1659 and 1630cm^{-1} (β -sheet structures).

Figure 2. PC-CVA model rebuilt using only absorbances at wavenumbers significant for bacterial fatty acids. The model used 12 PC scores (99.9% explained variance) and shows separation of *P. putida* exposed to propranolol (purple) from control cells (grey). Other exposures were not separated from the control, revealing that observed lipid alterations are specific to exposure to propranolol.

Figure 3. Altered metabolites in *P. putida* exposed to propranolol. As a visual method for assessing significance of metabolites as discriminating biomarkers the area under the ROC curve (AUC) is plotted versus p-value from the ANOVA analysis. If a metabolite has an AUC = 0.5 it is equally distributed between the two classes. A metabolite with an AUC = 1 is diagnostic of the class. Brown open circles denote fold increases, and grey open circles fold decreases, in metabolite concentration in exposed cells. Metabolites with an AUC > 0.7 and a p-value $< 1.77 \times 10^{-2}$ (the FWER threshold for a critical p-value equivalent to 0.05) are labeled. Some labels have been moved for clarity. Metabolites with an AUC > 0.85 and a p-value $< 1.77 \times 10^{-2}$ were considered significant and selected for correlation analysis.

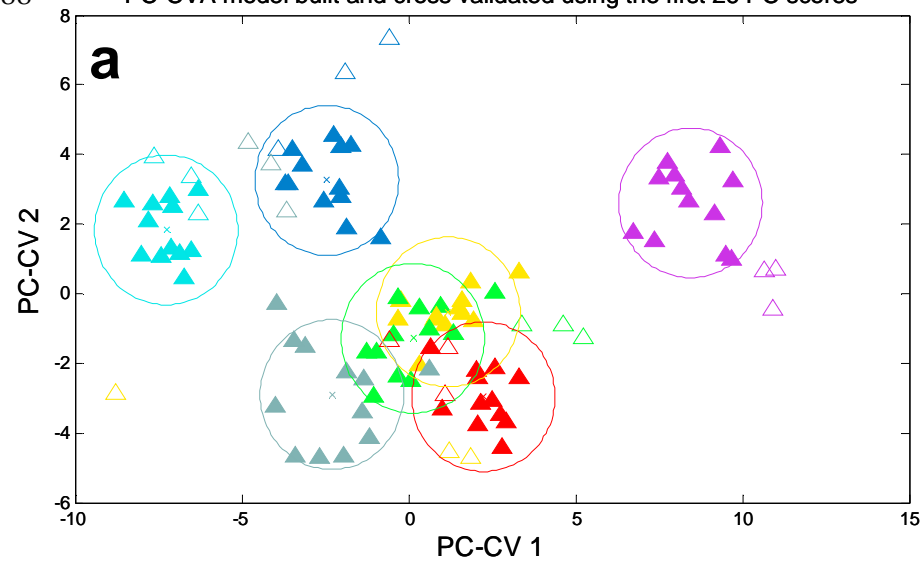
Figure 4. Spring embedded correlation plots showing correlation between 43 metabolites a) in cells exposed to water and b) in cells exposed to propranolol. Propranolol is correlated with energy-related metabolites: a sugar phosphate, trehalose and cellobiose; correlation is extended through metabolite 139, one of 3 metabolites with the lowest p-value whose concentration was raised from near the limit of detection, to succinic acid, a metabolite of the TCA cycle. In cells exposed to water trehalose and cellobiose are not correlated with succinic acid. Propranolol is also correlated with glycerol-3-phosphate, a precursor to the phosphatidyl group in glycerophospholipids.

Figure 5. Alterations in the concentration of energy related metabolites in *P. putida* exposed to propranolol identified from the ANOVA analysis. Trehalose and cellobiose, which have a role as energy reserves, were reduced in concentration in exposed cells, while succinic acid and sugar phosphates (increased in concentration in exposed cells).

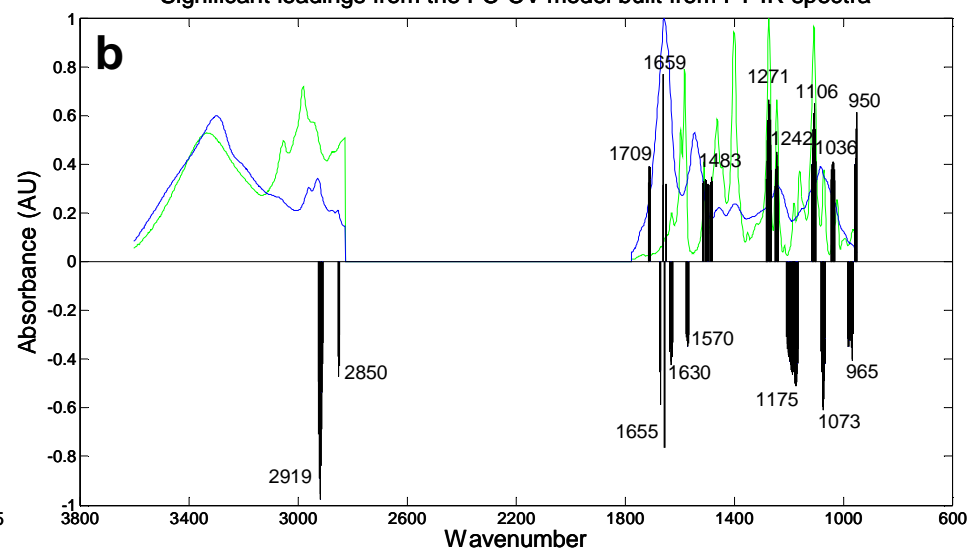
Figure 6. Alterations in ATP concentration in *P. putida* exposed to propranolol. The median ATP concentration prior to and 1h after exposure in cells exposed to water as a control and cells exposed to propranolol. p-values were calculated in an ANOVA analysis for the null hypothesis that the medians of the 2 groups are equal: the p-value for controls prior to exposure vs. controls 1 h after exposure = 1.2×10^{-1} and the p-value for exposed cells prior to exposure vs. 1 h after exposure = 1.2×10^{-5} , showing a significant difference in ATP concentration and energy demand in cells exposed to propranolol.

687 Figure 1

688 PC-CVA model built and cross validated using the first 26 PC scores



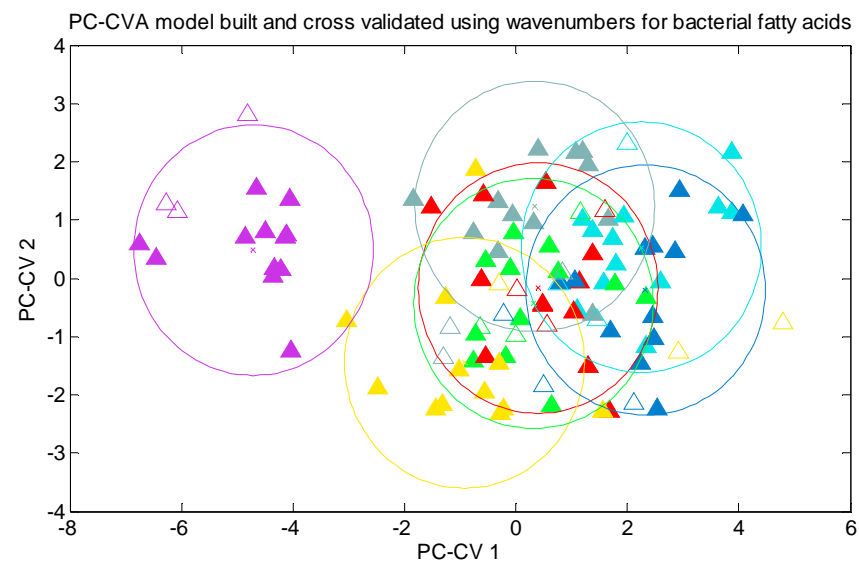
Significant loadings from the PC-CV model built from FT-IR spectra



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Figure 2

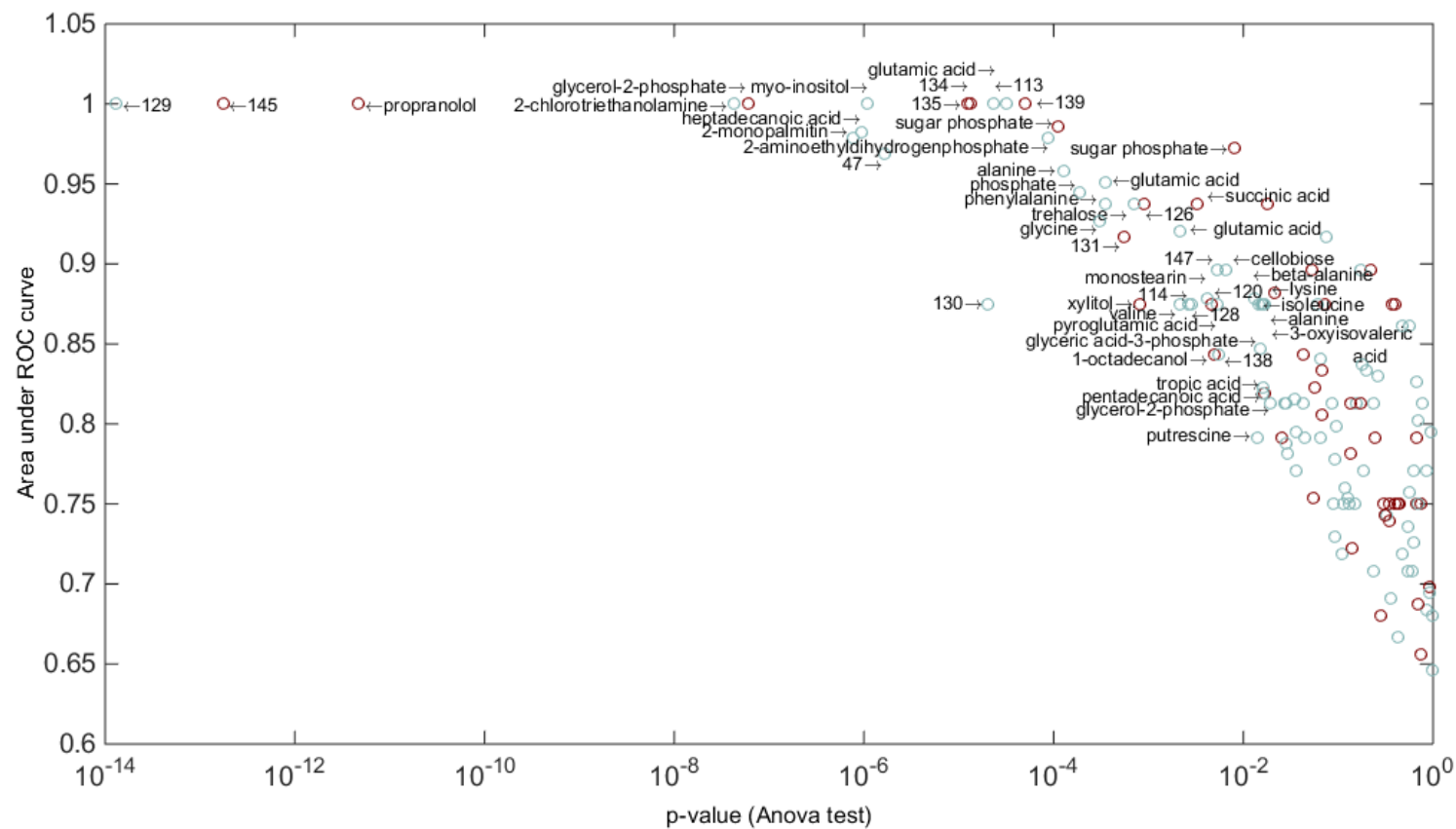
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Figure 3

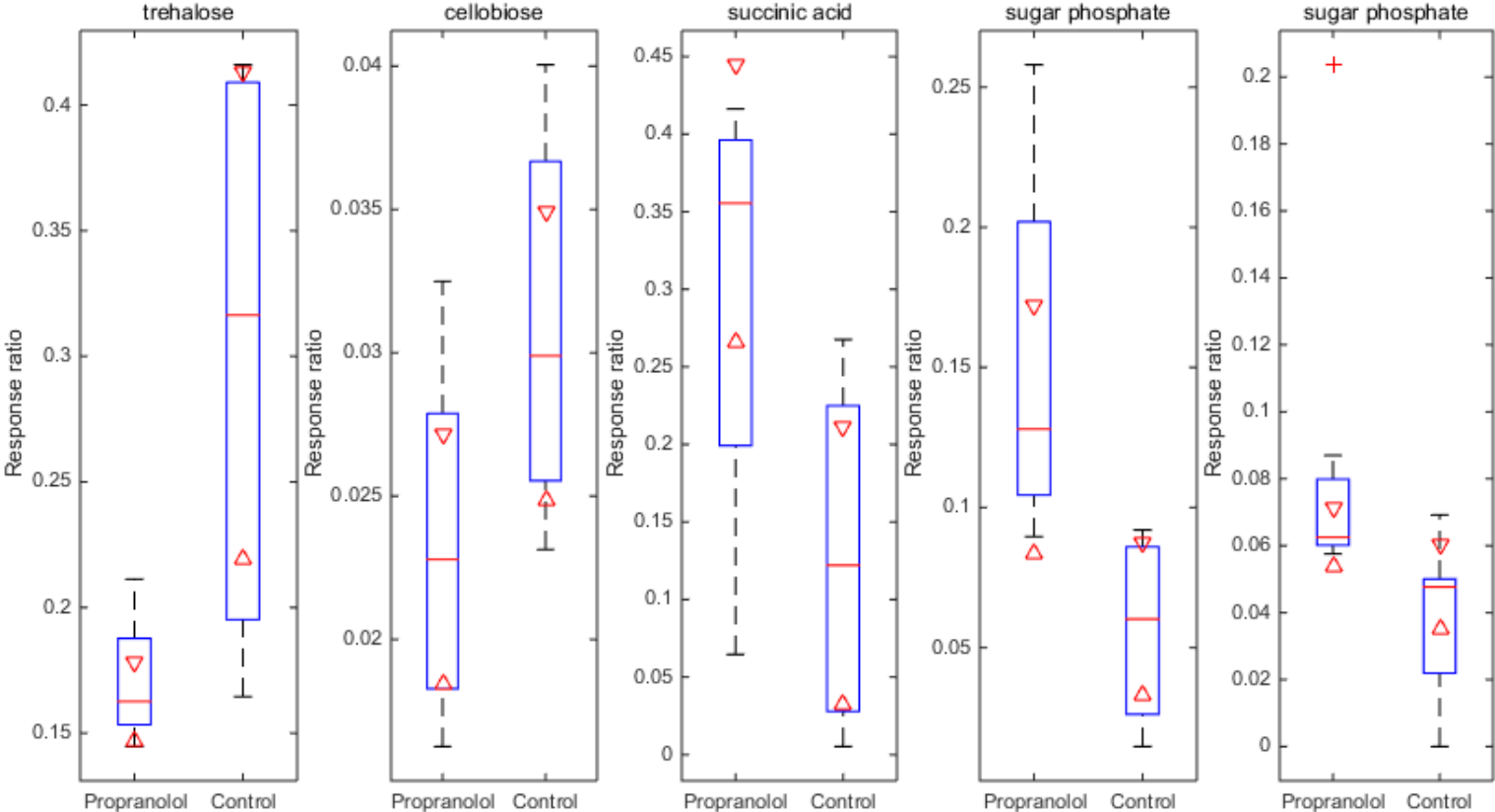
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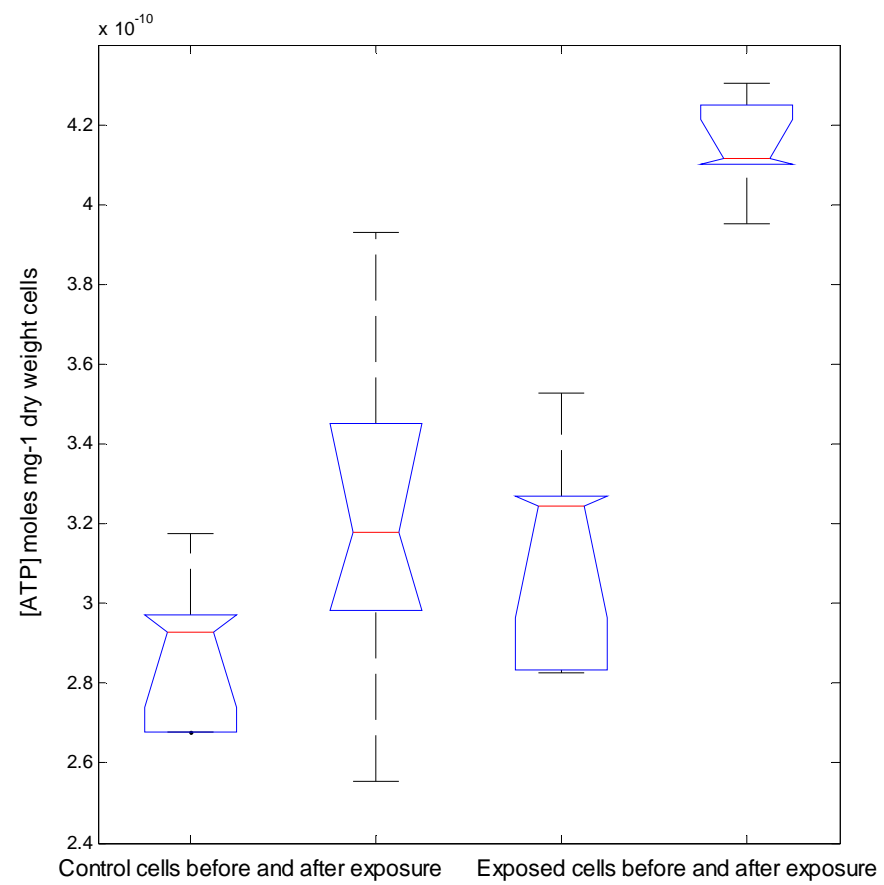
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700 Figure 6



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